See related commentary on pg 2707

ORIGINAL ARTICLE

Epithelial Cells in the Hair Follicle Bulge do not Contribute to Epidermal Regeneration after Glucocorticoid-Induced Cutaneous Atrophy

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One of the major adverse effects of glucocorticoid therapy is cutaneous atrophy, often followed by the development of resistance to steroids. It is accepted that epithelial stem cells (SCs) located in the hair follicle bulge divide during times of epidermal proliferative need. We determined whether follicular epithelial SCs and their transit amplifying progeny were stimulated to proliferate in response to the chronic application of glucocorticoid fluocinolone acetonide (FA). After first two applications of FA, keratinocyte proliferation in the interfollicular epidermis (IFE) and hair follicles was minimal and resulted in significant epidermal hypoplasia. We observed that a 50% depletion of the interfollicular keratinocyte population triggered a proliferative response. Unexpectedly, less than 2% of the proliferating keratinocytes were located in the bulge region of the hair follicle, whereas 82% were in IFE. It is known that cell desensitization to glucocorticoids is mediated via temporary decrease of glucocorticoid receptor (GR) expression. We found that GR expression was significantly decreased in IFE keratinocytes after each FA treatment. In contrast, many bulge keratinocytes retained GR in the nucleus. Our results indicate that bulge keratinocytes, including follicular SCs, are more sensitive to the antiproliferative effect of glucocorticoids than basal keratinocytes, possibly due to the incomplete process of desensitization.

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INTRODUCTION

The epidermis is a continuously renewing tissue that is replenished and repaired by epithelial stem cells (SCs). Several lines of evidence indicate that during high proliferative need, an important source of the proliferating cells in the epidermis are follicular epithelial SCs that reside within a specialized permanent segment of the outer root sheath of hair follicle known as the bulge (Lavker and Sun, 2000; Ito *et al.*, 2005a; Morasso and Tomic-Canic, 2005). Bulge epithelial SCs represent the most quiescent and long-lived epithelial cells in skin, and as such can be experimentally detected as label-retaining cells (LRCs)—cells that retain labeled DNA for months (Morris and Potten, 1999; Taylor *et al.*, 2000). Because of their high proliferative capacity that lasts throughout the lifetime of the organism, their multipotency, and strategic location within the specialized highly protected niche (Cotsarelis *et al.*, 1990; Morris and Potten, 1999; Blanpain *et al.*, 2004; Morris *et al.*, 2004), bulge epithelial SCs have been viewed for years as the major source of cells not only for the regeneration of hair follicles but also for regeneration and repair of epidermis. Indeed, these follicular epithelial SCs play an important role in the epidermal regeneration after physical or chemical removal of epidermis, superficial and full thickness skin wounding, or burns (Taylor *et al.*, 2000; Ito *et al.*, 2005a; Levy *et al.*, 2005). Recent studies indicated that there are other classes of cutaneous epithelial SC that are located in interfollicular epidermis (IFE) and possibly in sebaceous gland (Ghazizadeh and Taichman, 2001; Ito *et al.*, 2005a; Levy *et al.*, 2005; Horsley *et al.*, 2006).

Glucocorticoid hormones are potent inhibitors of keratinocyte proliferation and effective anti-inflammatory drugs, which have been widely used for the treatment of hyperproliferative and inflammatory skin disorders such as atopic dermatitis and psoriasis (reviewed in Perez *et al.*, 2001; Budunova *et al.*, 2003; Schoepe *et al.*, 2006). However, their chronic use is accompanied by adverse effects. One of the most prominent side effects of glucocorticoid therapy is skin atrophy that affects different skin components and compromises the barrier function of the skin. Glucocorticoid-induced skin atrophy is characterized by a reduction in epidermal thickness, a decreased number of keratinocytes, diminished stratum corneum intercellular lipid lamella, loss of ground substance, altered orientation and packing of collagen and elastin fibers combined with a decreased cellularity in dermis, elimination of subcutaneous fat, and loss of mast

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Abbreviations: BrdU, 5-bromo-2-deoxyuridine; FA, fluocinolone acetonide; GR, glucocorticoid receptor; IFE, interfollicular epidermis; LRCs, label-retaining cells; Mt1, Metallothionein 1; SCs, stem cells

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cells (Jablonska *et al.*, 1979; Lehmann *et al.*, 1983; Zheng *et al.*, 1984; Lavker *et al.*, 1986; Lubach and Kietzmann, 1988; Schoepe *et al.*, 2006).

The cellular response to glucocorticoids is mediated through the highly specific glucocorticoid receptor (GR) (Hollenberg *et al.*, 1985; Beato *et al.*, 1995). Non-active GR is bound to the heat-shock protein complex, and is sequestered in the cytoplasm (Beato *et al.*, 1995; Yudt and Cidlowski, 2002; De Bosscher *et al.*, 2003). Following ligand binding, the GR dissociates from the chaperones and forms homodimers, which enter the nucleus. There are two major mechanisms of gene regulation by the GR (De Bosscher *et al.*, 2003). The first is the positive transcriptional regulation of genes (transactivation) requiring the binding of the GR homodimer to DNA sequences called glucocorticoid-response elements in the promoter or enhancer region of glucocorticoid-responsive genes. The second is negative gene regulation (trans-repression) mediated via GR binding to negative glucocorticoid-response elements or via cross-talk with other transcription factors, including activator protein-1 (AP-1), NF-κB, and others, resulting in the inhibition of the partner transcription factor activity (Adcock, 2001; De Bosscher *et al.*, 2003; Necela and Cidlowski, 2004; Schacke et al., 2004; Yemelyanov *et al.*, 2007). Recently an additional mechanism of indirect gene regulation by the GR was discovered, where GR blocks mitogen-activated protein kinases (Kassel *et al.*, 2001; Imasato *et al.*, 2002; Bruna *et al.*, 2003; Necela and Cidlowski, 2004; Yemelyanov *et al.*, 2007). We and others showed that GR is expressed in rodent and human IFE (Karstila *et al.*, 1994; Budunova *et al.*, 1997; Perez *et al.*, 2001; Ito *et al.*, 2005b). However, GR expression in a bulge area of hair follicles has not been well studied.

To determine whether bulge keratinocytes (including follicular epithelial SCs) are involved in epidermal repair after development of steroid-induced skin atrophy, we studied the GR expression in the bulge and the kinetics of bulge-located keratinocyte proliferation, as well as IFE, when 15, 30, and 50% epidermal hypoplasia was achieved. We report herein that bulge keratinocytes express GR and are sensitive to glucocorticoid-induced growth inhibition. Unexpectedly, bulge keratinocytes developed resistance to glucocorticoids more slowly than basal keratinocytes, and did not significantly contribute to the reparation of epidermis after glucocorticoid-induced skin atrophy.

RESULTS

GR is expressed in hair follicular bulge keratinocytes

It was shown that GR is prominently expressed in IFE (Karstila *et al.*, 1994; Budunova *et al.*, 1997; Perez *et al.*, 2001; Ito *et al.*, 2005b). However, the GR expression in the bulge area of hair follicles, a region that is enriched in follicular epithelial SCs, has not been well studied. To assess GR expression in this population of keratinocytes as well as in basal keratinocytes, we isolated bulge keratinocytes and basal keratinocytes from six mice, whose hair follicles were in the telogen stage of the hair cycle, by FACS analysis using previously reported bulge surface markers CD34 and α6-integrin (Trempus *et al.*, 2003; Blanpain *et al.*, 2004; Tumbar *et al.*, 2004). Two-color FACS analysis allowed us to isolate a discrete, highly homogenous fraction of bulge keratinocytes: the purity of CD34$^+$/α6-integrin$^+$-sorted keratinocytes was $\sim$90%, as confirmed by post-sort FACS analyses (Chebotaev *et al.*, 2007). We used data of our bulge/IFE keratinocyte gene array (Chebotaev *et al.*, unpublished data) to compare the expression of GR in these two keratinocyte populations isolated from skin of the same animals. On gene array, the expression of Nr3c1 (gene symbol for GR) was 1.6-fold higher (P-value = 0.0002) in bulge keratinocytes. We confirmed these data by quantitative real-time PCR that revealed that the expression of GR mRNA in bulge cells was 30–40% higher.

To study the expression of GR protein in bulge keratinocytes, we used double immunofluorescence of skin sections with anti-GR and anti-CD34 Abs, as CD34 expression defines bulge boundaries. We found that GR was equally expressed in IFE, in the upper part of hair follicle, and in the bulge region of the hair follicle (Figure 1). GR-related immunoreactivity was localized not only in cytoplasm but also in the nuclei of keratinocytes, especially in the hair follicles (Figure 1). This partial nuclear localization of GR is possibly due to receptor activation by endogenous glucocorticoids synthesized in hair follicles (Ito *et al.*, 2005b).

GR expression and function in keratinocytes during topical application of fluocinolone acetonide

One of the mechanisms of cell desensitization to the effect of glucocorticoids is mediated via a negative feedback loop that results in the temporary decrease of GR expression upon treatment with hormone (Zong *et al.*, 1990; Oakley and Cidlowski, 1993; Budunova *et al.*, 1997). Thus, to choose the most effective treatment regimen to produce a glucocorticoid-induced epidermal hypoplasia, we identified the time of GR expression recovery after topical application of the synthetic glucocorticoid fluocinolone acetonide (FA). Western blotting (Figure 2a) indicated that the level of GR protein was significantly decreased 24 hours after FA application, but returned to control level 48 hours after treatment. Thus, we have chosen the intermittent regimen of treatment and applied FA topically every 48 hours for our experiments.

To further confirm the validity of the intermittent regimen, we assessed the GR function in keratinocytes at the beginning and end of FA applications. For this, we performed Northern blot analysis of expression of the endogenous glucocorticoid-responsive gene Metallothionein I (*Mt1*). In our previous work we showed that *Mt1* is a reliable endogenous GR reporter, whose expression strongly depends on GR activity (Budunova *et al.*, 1997). We found that *Mt1* expression was induced at an equal level after first and fourth applications of FA (Figure 2b), indicating that GR was fully functional during the course of skin treatment.

Effect of intermittent skin treatment with glucocorticoid on skin morphology, epidermal cellularity, and keratinocyte proliferation

In the skin of F1 C57BlxDBA control mice (8 weeks old) treated with vehicle, the 5-bromo-2-deoxyuridine (BrdU)

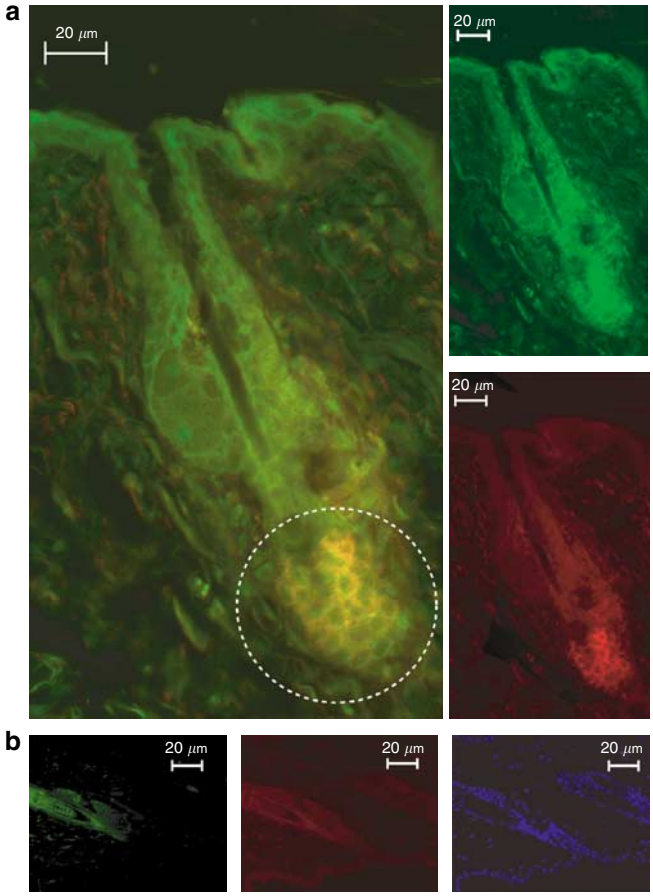

Figure 1. Coexpression of GR and CD34 marker in bulge keratinocytes in hair follicle. (**a**) Skin sections from control animals were double immunostained with anti-GR Ab (green) and anti-CD34 Ab (red). Sections were analyzed by fluorescent microscope. The left panel shows coexpression of GR and CD34 in bulge keratinocytes (bulge area is circled). The top right panel shows GR expression in hair follicle and IFE; the bottom right panel shows CD34 expression in hair follicle and IFE. (**b**) Skin sections from the same animals were stained only with FITC- and Cy3-conjugated secondary Abs (left and middle panels, negative control), and counterstained with DAPI to identify the nuclei. Note: GR is well expressed in bulge and IFE, and is localized both in the nuclei and in the cytoplasm of keratinocytes. In the bulge GR is coexpressed with the marker CD34.

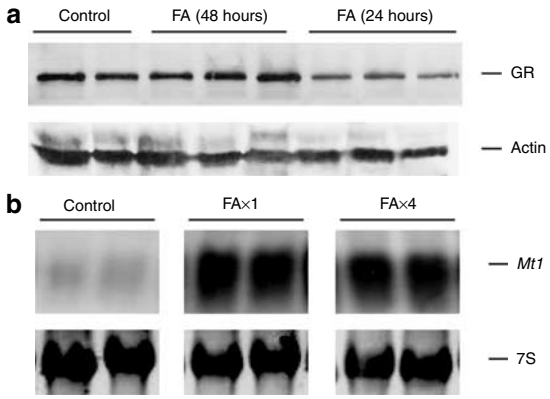

Figure 2. Expression and function of GR in epidermis after glucocorticoid treatment. (**a**) Western blot analysis of GR expression. Animals were treated with FA or acetone (control) once. The whole cell protein extracts were prepared from epidermis 24 and 48 hours after FA application, and used for Western blot analysis of GR expression. Probing with anti-α-actin Ab was used as control for protein loading. (**b**) Northern blot analysis of expression of GR-responsive gene *Mt1*. Animals were treated with FA every second day (intermittent regimen). Twenty-four hours after the first and fourth FA applications, total RNA from epidermis was isolated and used for Northern blot analysis of *Mt1* expression. Probing with 7S ribosomal RNA was used to control RNA loading. *Note: Mt1* expression was induced at an equal level after first and fourth applications of FA indicating that GR was fully functional during the course of skin treatment with FA.

labeling index was 2.5%. This correlates well with the usual 2–4% proliferation rate of basal keratinocytes in the IFE of adult mouse and human epidermis (Budunova *et al.*, 1997; Jensen and Lavker, 1999). The proliferation rate of keratinocytes in hair follicles was ∼1.5% (∼1 BrdU-positive cell/follicle), as mice follicles were in the telogen (resting) stage of the hair cycle.

After the first and second FA applications, the proliferation of keratinocytes in the IFE and in hair follicles was almost completely blocked. The proliferative rate of the basal keratinocytes in IFE decreased to 0.3%, and to 0.1% after the first and second FA applications, respectively, and there were almost no BrdU-positive cells in hair follicles (see below). Likewise, the proliferation of cells in the sebaceous gland was also strongly inhibited (data not shown).

The dramatic changes in proliferation of keratinocytes and other skin cells during the intermittent regimen of glucocorticoid treatment resulted in a significant skin atrophy manifested by the loss of thickness of all the cutaneous compartments. After the fourth FA application, the IFE was markedly thin and was comprised of a thin, loosely formed basal layer, and in some places by a basal and a granular layer (Figure 3). The keratinocytes in basal compartment were flat with elongated nuclei, and were oriented horizontally along the basement membrane, resembling the differentiated keratinocytes in the upper spinous/granular layers in control skin. The hair follicles at this time of FA treatment were in the telogen phase of hair cycle (Figure 3), and no extension of the secondary hair germ was observed. We also noticed a regression of the sebaceous glands.

We measured the epidermal thickness and epidermal cellularity (the number of basal keratinocytes/mm of IFE) during the course of treatment and found changes that were consistent with their phenotype. The thickness of epidermis and number of basal keratinocytes were reduced after the second FA treatment by 30 and 20%, respectively (Figure 4a). After the fourth FA application, a 50% decrease in both epidermal thickness and basal keratinocyte number was noted (Figure 4a).

At 50% epidermal hypoplasia (50% depletion in the number of basal keratinocytes), some keratinocytes and other skin cells such as sebocytes started to develop resistance to the growth inhibitory effect of glucocorticoids and began to proliferate (Figure 5); a phenomenon known as tachyphylaxis.

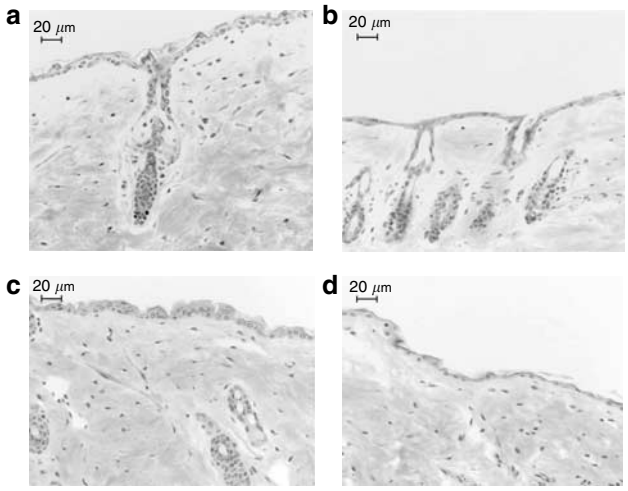

Figure 3. Topical treatment with glucocorticoid FA induced strong skin atrophy in mice. Animals were treated with acetone (control) or FA (intermittent regimen), and skin was harvested 24 hours after the last FA application. Skin sections were stained with hematoxylin/eosin. (**a**, **c**) Control skin after acetone treatment. (**b**, **d**) Skin after FA treatment. Note that intermittent regimen of FA treatment resulted in significant skin atrophy.

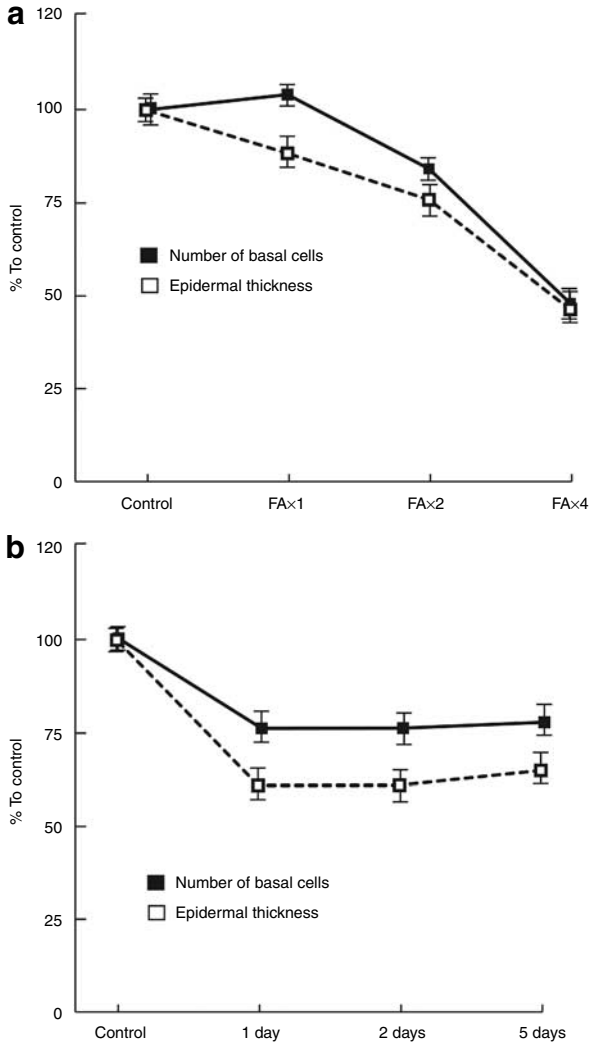

Figure 4. Quantitative analysis of epidermal hypoplasia induced by FA in mouse skin. Animals were treated with acetone (control) or with FA. Skin was harvested, stained with hematoxylin/eosin, and the number of basal cells and the thickness of epidermis were determined as described in Materials and Methods. A total of 40–50 microscopic fields of view were examined in each skin sample. A total of three to four samples from individual animals in each experimental group were used for analysis. The changes in FA-treated epidermis were calculated as percentage to control, and the results of representative experiment are presented as mean ± SD. (**a**) Intermittent regimen of treatment. Skin was harvested 24 hours after the first, second, and fourth FA applications. (**b**) Continuous regiment of treatment. Skin was harvested 1, 2, 3, 4, and 5 days after the last FA application. Note that intermittent regimen of FA treatment resulted in more pronounced epidermal hypoplasia.

It is known that follicular epithelial SCs cells and their progeny are important for repopulating the epidermis after injury (Taylor *et al.*, 2000; Ito *et al.*, 2005a; Levy *et al.*, 2005). Thus, we expected to find an induction of proliferation of bulge keratinocytes in response to the significant glucocorticoid-induced cutaneous hypoplasia. Unexpectedly, analysis of >300 BrdU-positive keratinocytes in skin sections of animals treated with FA for four times revealed that more than 80% of proliferating keratinocytes were located in IFE (Figure 6a and b). In contrast, only 16% of proliferating cells were found in the upper part of hair follicle, and less than 2% in the bulge of hair follicles. Moreover, the proliferation rate of keratinocytes in IFE after the fourth application of FA returned to the control level control (3.2 ± 0.4% in FA-treated skin, and 2.4 ± 0.3% in control skin; Figure 6a). At the same time, proliferation of keratinocytes in hair follicles reached only 25% of control proliferation levels after the fourth FA application (Figure 6a). This result suggests that cells in hair follicle are more sensitive to the growth inhibitory effect of corticosteroids, and gain resistance to hormone with the significant delay.

We initially expected that the proliferation of follicular SCs would be stimulated during the restoration of the epidermis following glucocorticoid-induced cutaneous atrophy. Thus, we assessed bulge epithelial SC proliferation at the time of 50% epidermal hypoplasia using a previously described double labeling approach (Lehrer *et al.*, 1998; Taylor *et al.*, 2000). For this we injected newborn pups subcutaneously with BrdU, twice a day for 3 days. This protocol resulted in ~100% of the keratinocytes incorporating BrdU 24 hours after the last injection. Following an 8-week chase, the rapidly cycling TA cells in the upper part of outer root sheath and in IFE divided, diluted the BrdU label, and underwent terminal differentiation. At the same time, most of the quiescent BrdU-stained cells (LRCs) resided in the bulge region of the hair follicles. To assess the proliferation of LRC in adult skin, mice bearing LRCs were injected with [^{3}H]thymidine 1 hour before skin was harvested. [^{3}H]Thymidine was mostly incorporated by keratinocytes in IFE, where hardly any LRCs were localized. We did not find any proliferating ([^{3}H]thymidine positive) LRCs in the bulge area after four FA applications (data not shown).

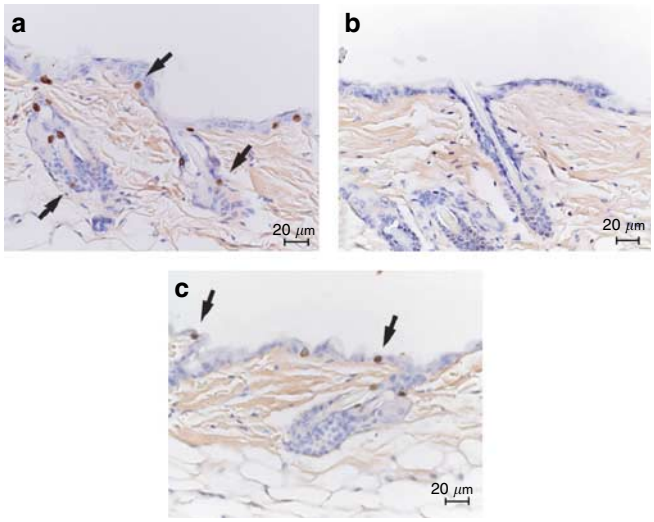

Figure 5. Effect of chronic skin treatment with FA on keratinocyte proliferation. Animals were treated with acetone (control) or with FA (intermittent regimen of treatment) and injected intraperitoneally with BrdU 1 hour before skin was harvested. BrdU-positive cells were identified by immunostaining. (**a**) Control; (**b**) FA × 1 (24 hours after first FA application); (**c**) FA × 4 (24 hours after fourth FA application). BrdU-positive cells are indicated by arrows. Note that in FA × 4-treated skin, most BrdU-positive keratinocytes are localized in the IFE.

Effect of continuous skin treatment with glucocorticoid on epidermal cellularity and keratinocyte proliferation

To ensure that we did not miss the optimal time of bulge keratinocyte proliferation during the intermittent treatment regimen with FA, we treated mice with FA for four consecutive days, and studied keratinocyte proliferation 1, 2, 3, 4, and 5 days after the last steroid application. The epidermal hypoplasia that resulted from this continuous treatment was less pronounced than hypoplasia induced by intermittent FA treatment. After the last glucocorticoid application, epidermal thickness was reduced by 35% when FA was applied every 24 hours, compared to a 55% reduction when FA was applied every other 24 hour (Figure 4b). Accordingly, the number of basal keratinocytes was reduced by 25% after the daily treatment with FA, and by 50% after intermittent treatment with FA. This finding probably reflects that fact that GR expression is downregulated when FA applied every 24 hours (Figure 2a).

The epidermal hypoplasia induced by continuous FA treatment was persistent. Five days after the last FA application, the epidermal thickness was still reduced by 35% compared to untreated epidermis (Figure 4b). Apparently a 35% reduction in epidermis thickness and a 25% reduction in basal layer cellularity (Figure 4b) were not sufficient to induce keratinocyte proliferation. We did not observe any BrdU-labeled keratinocyte 1–3 days after the last FA application. However, because the epidermal hypoplasia was persistent, activation of keratinocytes to proliferate eventually occurred. A few BrdU-positive keratinocytes were observed on the fourth day after FA treatment was stopped, and on the fifth day after the treatment, a significant number of keratinocytes were in S-phase. The analysis of >170

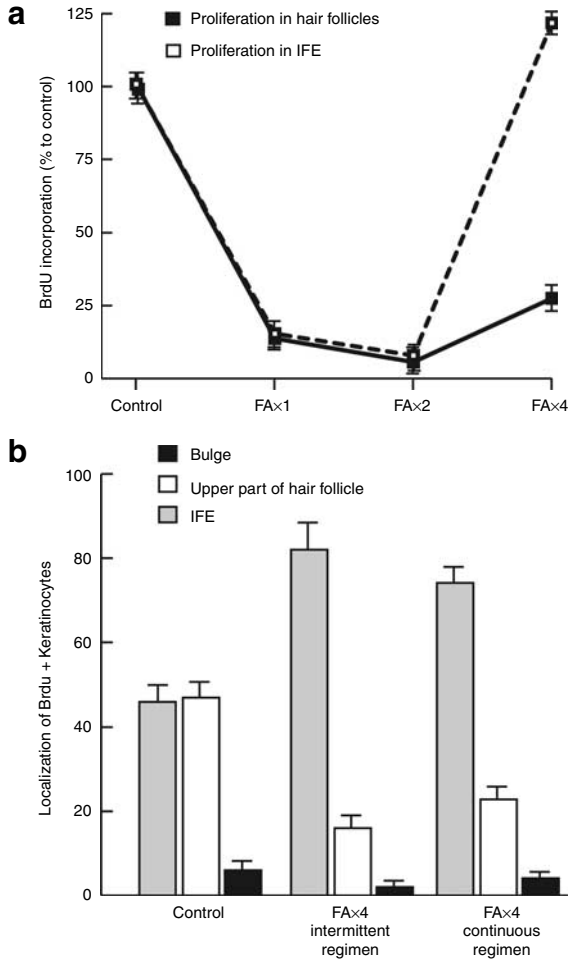

Figure 6. Preferential localization of BrdU-positive keratinocytes in IFE at the stage of resistance to FA. (**a**) Number of BrdU-positive keratinocytes in hair follicles and in IFE during the intermittent regimen of FA treatment. Animals were treated as indicated in legend for Figure 5a, and injected intraperitoneally with BrdU, 1 hour before skin was harvested. BrdU-positive keratinocytes were identified by immunostaining, and their localization was determined. The number of BrdU-positive keratinocytes in IFE and hair follicles is presented as percent to the number of BrdU-positive keratinocytes in IFE and hair follicles in control skin. The mean ± SD was calculated for four individual skin samples in one representative experiment. In each sample, a total of 40–50 individual fields of view were examined. (**b**) Localization of BrdU-labeled keratinocytes did not depend on the regimen of skin treatment with FA. Animals were treated with acetone (control) or FA. Skin was harvested 24 hours after the last FA treatment (intermittent regimen) or 5 days after the last FA treatment (continuous regimen). The number of BrdU-positive keratinocytes in IFE, upper part of hair follicle, and the bulge or hair follicles is presented as percentage to the total number of examined BrdU-positive keratinocytes. The mean ± SD was calculated for four individual skin samples in one representative experiment. In each sample, a total of 40–50 individual fields of view were examined. Note that ~70–85% of proliferating keratinocytes are located in IFE after both regimens of FA treatment.

BrdU-positive keratinocytes on skin sections of animals treated continuously with FA again showed that ~70% of proliferating keratinocytes were located in IFE, ~24 % were located in the upper part of hair follicle, and less than 5% were found in the bulge (Figure 6b).

Differential regulation of GR by glucocorticoid in keratinocytes in hair follicle bulge and IFE

As we have discussed above, one of the well-known mechanisms of keratinocyte desensitization to glucocorticoids is mediated via the temporary decrease of GR expression. In our previous and current experiments, we revealed downregulation of GR expression at mRNA and protein levels (Budunova *et al.*, 1997; Figure 2a). For those studies we used RNA and protein samples from epidermis physically separated from dermis by scraping (see Materials and Methods). This procedure allows isolation of keratinocytes from IFE and the upper part of hair follicles but not from the bulge. Thus, to understand the dynamics of GR expression in bulge keratinocytes after FA treatment, we performed immunostaining. The results of immunostaining revealed the striking difference in GR regulation by FA in two keratinocyte populations. Indeed, in control skin GR was well expressed in follicular and IFE keratinocytes and was localized in cytoplasm as well as in the nuclei (Figure 7a). Twenty-four hours after the first FA application, the overall expression of GR in both keratinocyte populations was significantly decreased compared to control skin (Figure 7c). This decrease correlates well with the data of Western blotting (Figure 2a). Surprisingly, in comparison to IFE keratinocytes, in which both nuclear and cytoplasm GR expression was reduced, many bulge keratinocytes retained GR in the nuclei (black arrows in Figure 7c, insets). This finding suggests that GR desensitization was less complete in the bulge than in IFE. The immunostaining of skin treated with FA four times (intermittent regimen, 24 hours after the last treatment) further confirmed this observation (Figure 7d).

DISCUSSION

Recent studies clearly indicated that there are several classes of epithelial SCs in skin. The primary source of multipotent SCs is the follicular epithelial SCs located in the bulge (reviewed by Blanpain and Fuchs, 2006; Ito *et al.*, 2005a; Morasso and Tomic-Canic, 2005). The capability of isolated bulge cells to reconstitute both hair follicle and epidermis has led to the conclusion that these cells are the ultimate source for the regeneration and maintenance of both hair follicles and IFE (Taylor *et al.*, 2000; Blanpain *et al.*, 2004; Morris *et al.*, 2004; Tumbar *et al.*, 2004). Indeed, these cells have been shown to play an important role in the repair of the epidermis after physical removal, wounding, and burns (Taylor *et al.*, 2000; Ito *et al.*, 2005a; Levy *et al.*, 2005).

The secondary source are epidermal SCs located throughout the basal layer of IFE, that have an epidermal-regenerative capacity (Cotsarelis *et al.*, 1999; Li *et al.*, 2004), but are somewhat less multipotent than the bulge SCs (Morasso and Tomic-Canic, 2005; Blanpain and Fuchs, 2006). Epithelial SC lineage studies using genetic approaches clearly showed that bulge keratinocytes (SCs) do not usually contribute to the maintenance of the normal IFE (Ghazizadeh and Taichman, 2001; Ito *et al.*, 2005a; Levy *et al.*, 2005).

The experiments presented here were designed to determine whether (i) bulge keratinocytes are stimulated to proliferate and thus are involved in the restoration of epidermal homeostasis after the development of glucocorticoid-related skin atrophy, and (ii) what level of epidermal cell loss triggers follicular SC proliferation. Using different regimens of glucocorticoid treatment, we were able to achieve incremental 15, 30, and 50% epidermal hypoplasia, and

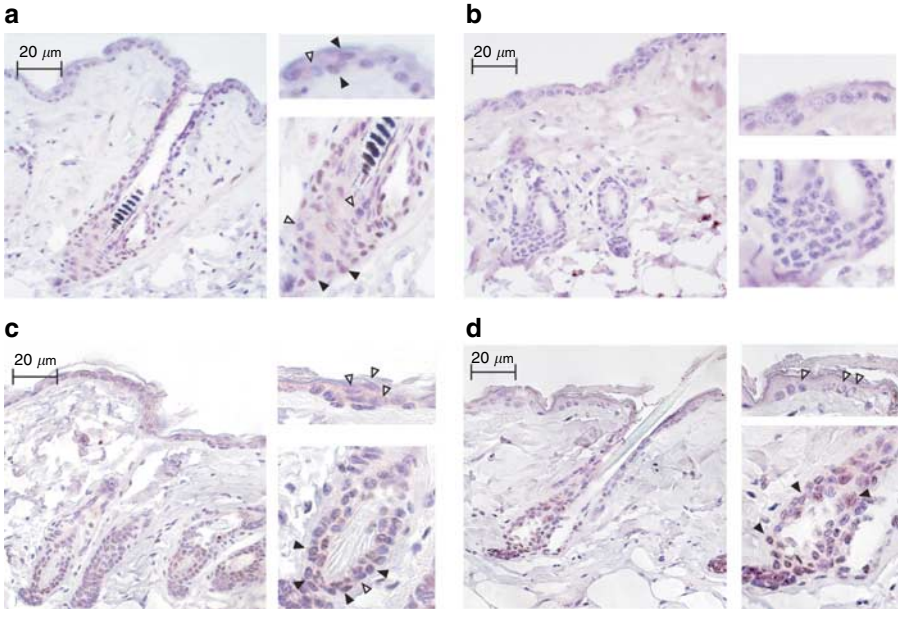

Figure 7. Changes in GR expression in different keratinocyte populations after FA treatment. Animals were treated with acetone (control) or FA (intermittent regimen of treatment as in legend for Figure 5a). Skin was harvested 24 hours after last FA application, fixed, and used for GR immunostaining. (**a**) Control; (**c**) FA × 1 (24 hours after first FA application); (**d**) FA × 4 (24 hours after fourth FA application). (**b**) negative control for GR immunostaining (acetone-treated skin was incubated with rabbit serum instead of anti-GR Ab). GR-positive nuclei are indicated by black arrows; GR-negative nuclei are indicated by white arrows. Note: in control skin, GR has both nuclear and cytoplasmic localizations in bulge and IFE keratinocytes. In FA-treated skin, most keratinocytes in IFE do not express GR in the nucleus, while bulge keratinocytes retain GR in the nucleus.

showed that only a 50% depletion of the interfollicular keratinocyte population triggers a proliferative response. Unexpectedly, more than 80% of the keratinocytes that entered S-phase in response to epidermal hypoplasia were located within IFE, possibly indicating the involvement of the epidermal as opposed to the follicular epithelial SCs in this process. We hypothesize that BrdU-positive keratinocytes that started to proliferate in IFE are either epidermal SCs or their early TA cell progeny. Unfortunately, these epidermal SCs are much less well characterized than follicular epithelial SCs, in terms of the surface marker expression. Thus, direct confirmation of this idea will require the development of reliable epidermal SC markers.

Little is known about the signals that induce epithelial SCs to proliferate during epidermal renewal and repair. For example, during skin wounding, multiple pro-proliferative cytokines and growth factors are released from the keratinocytes and fibroblasts, including IL-1, tumor necrosis factor-a, epidermal growth factor (EGF), and others (Morasso and Tomic-Canic, 2005). Glucocorticoids are known to inhibit wound healing via different mechanisms, including the inhibition of keratinocyte proliferation (Lehmann *et al.*, 1983; Morasso and Tomic-Canic, 2005; Schoepe *et al.*, 2006). The negative cross-talk between GR and pro-proliferative signaling mediated by cytokines and growth factors, including EGF, is also well known (Morasso and Tomic-Canic, 2005; Yemelyanov *et al.*, 2007). Thus, even though the role of specific pro-proliferative factors in the initiation of follicular and epidermal SC proliferation remains to be investigated, it is reasonable to assume that the negative interaction between GR and pro-proliferative factor signaling may underlie the inhibition of SC proliferation, and contribute to the inhibition of wound healing by corticosteroids.

In the light of the strong growth inhibitory effect of glucocorticoids on keratinocytes, the experimental design used in our experiments to induce proliferation of epithelial SCs was different compared to wounding or mechanical removal of epidermis. In our experiments, epidermal SCs not only respond to the dramatic decrease in epidermal cellularity but also overcome the growth inhibitory effect of steroids.

One of the known temporary mechanisms of desensitization of cells to the effect of glucocorticoids is mediated via inhibition of GR expression upon treatment with hormone (Zong *et al.*, 1990; Oakley and Cidlowski, 1993; Budunova *et al.*, 1997). Our results indicate that in contrast to IFE, GR downregulation in bulge keratinocytes is incomplete. Nuclear localization of GR suggests that it remains active as a transcription factor and continues to negatively control proliferation of bulge keratinocytes. However, the experimental proof of the above statement requires the direct assessment of GR functional activity and evaluation of downstream GR target genes in the specific keratinocyte populations. Overall, we hypothesize that the inability of bulge keratinocytes, including follicular epithelial SC, to develop tachyphylaxis to glucocorticoids is partially the consequence of insufficient mechanisms of desensitization in this cell population.

Our recent results obtained in Keratin5.GR transgenic animals provide additional evidence that follicular epithelial SCs are highly sensitive to GR signaling. We found that the number of putative follicular epithelial SCs and their clonogenicity was strongly reduced in Keratin5.GR transgenic animals that express high level of GR in basal keratinocytes in IFE and in the bulge (Chebotaev *et al.*, 2007). It is interesting that in contrast, the number of basal keratinocytes and their proliferation in intact adult skin of K5.GR mice did not change compared to wild-type littermates (Chebotaev *et al.*, 2007).

There are other physiological and pathological conditions in skin that are associated with the development of glucocorticoid resistance. For example, keratinocytes in neonatal skin and skin tumors are resistant to growth inhibition by glucocorticoids (Slaga *et al.*, 1978; Budunova *et al.*, 1997). It is interesting that in all these cases the development of resistance to steroids was associated with intensive proliferation and expansion of the basal keratinocyte compartment (which obviously involved proliferation of normal and transformed epithelial SCs and their early progeny). These data highlight the important role of GR in the regulation of proliferation of cutaneous epithelial SCs. They also suggest that to improve the overall outcome of treatment with glucocorticoids, the optimization of the treatment regimen based on the dynamics of GR expression and the dynamics of development of glucocorticoid resistance (tachyphylaxis) should be considered.

In conclusion, our results suggest that bulge keratinocytes do not significantly contribute to the restoration of the epidermis after glucocorticoid-induced skin atrophy, and highlight the potential role of SCs that reside in IFE as a major source for the regeneration of the epidermis during the steroid-induced hypoplasia. Thus, our results support the recent evidence that epidermal and follicular epithelial SCs are distinct cell populations with different properties and roles in the skin homeostasis and regeneration. The understanding of the effects of glucocorticoid on different classes of cutaneous epithelial SCs, and the mechanism(s) of SC resistance to glucocorticoids will allow the development of therapeutic approaches to treat/prevent glucocorticoid-induced skin atrophy, which is one of the major adverse effects of chronic steroid usage.

MATERIALS AND METHODS

Animals and treatments

B6D2 (F1 C57Bl × DBA) females were obtained from Jackson Laboratory (Bar Harbor, ME). Seven to eight weeks old animals in the telogen stage of the hair cycle were shaved, and treated 3 days later with glucocorticoid FA (Sigma, St Louis, MO). FA was applied topically (5 μg/animal) in 200 μl acetone. Control animals were treated with acetone only.

To study the effect of FA on skin atrophy and keratinocyte proliferation, we used two different regimens. During the intermittent regimen, animals were treated with FA four times every second day and killed by CO_2 inhalation followed by cervical dislocation 24 hours after the first, second, and fourth FA applications. During the continuous regiment, animals were treated with FA

four times for four consequent days, and killed 1, 2, 3, 4, and 5 days after the last FA application. All animals were injected intraperitoneally with BrdU (Sigma, St Louis, MO) solution in phosphate-buffered saline (50 μg/g of animal weight) 1 hour before they were killed. All animal experiments were performed in compliance with ACUC protocol approved by the Northwestern University Animal Care and Ethics Committee.

Morphometric analysis of epidermis

Mice were treated with FA using intermittent and continuous regimens, as described above. Dorsal skin was harvested, fixed in formalin, processed to 5 μm paraffin sections, stained with hematoxylin/eosin, and used for morphometric analyses. The number of basal keratinocytes was calculated per 1 mm of IFE. The thickness of epidermis was assessed using Axioplan2 microscope software (Carl Zeiss, Oberkochen, Germany). A total of 40–50 individual fields of view were examined in each skin sample, with three to four samples from individual animals in each experimental group. Ten measurements of epidermis thickness were performed randomly in each field of view. The number of basal keratinocytes and the epidermal thickness in FA-treated animals are presented as the percentage of those parameters in control animals.

Immunostaining

Formalin-fixed mouse skin samples were used for BrdU, GR, and CD34/GR double immunostaining. After antigen retrieval (10 minutes at 90°C in citric buffer, pH 6.0), the tissues were blocked with 10% horse serum in phosphate-buffered saline. For BrdU staining, tissues from control and FA-treated animals were incubated with mouse monoclonal anti-BrdU Ab (BD Biosciences, San Jose, CA), followed by biotinylated secondary anti-mouse IgG from Mouse-on-mouse kit and ABC reagent from Standard VECTASTAIN® Elite ABC kit (Vector Laboratories, Burlingame, CA). Immunostaining was visualized with DAB chromogen (Vector Laboratories, Burlingame, CA), and tissues were counterstained with Gill's hematoxylin (Vector Laboratories, Burlingame, CA). The number of BrdU-positive cells was calculated as indicated in the figure legends. For GR staining, tissues were incubated with rabbit anti-GR Ab (Santa-Cruz Biotechnology, Pasadena, CA) followed by the procedures described above. For CD34/GR double immunofluorescence, tissues from control animals were incubated with rabbit anti-GR Ab (Santa-Cruz Biotechnology, Pasadena, CA) and rat anti-mouse CD34 Ab (BD Pharmingen, San Diego, CA), followed by Cy3-conjugated donkey anti-rat and FITC-conjugated goat anti-rabbit secondary Abs (both from Jackson ImmunoResearch Laboratory Inc., West Grove, PA). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) to identify the nuclei. Immunofluorescence was analyzed using a fluorescent microscope (Axioplan2, Carl Zeiss, Germany).

Detection of proliferation of LRCs by double labeling approach

To assess the proliferation of putative SCs in the bulge, we used a double labeling approach as described previously (Lehrer *et al.*, 1998; Taylor *et al.*, 2000). To tag the LRCs, we injected newborn mice (postnatal day 3–5) subcutaneously with BrdU (50 μg/g body weight), twice daily for 3 days. Eight weeks later, BrdU-labeled mice were used for the intermittent FA treatment (as described above) followed by the detection of proliferation of LRC. For this purpose the FA-treated animals were injected with tritiated thymidine (H^3TdR, 10 μCi/g body weight) 24 hours after the first and the last FA application and killed 1 hour following injection. Dorsal skin was harvested, fixed in 70% ethanol–glycine solution, and processed to 5 mm paraffin sections. Slides were stained with anti-BrdU Ab and alkaline phosphatase-labeled secondary Ab (both from Boehringer, Indianapolis, IN), followed by visualization with red substrate (Vector Laboratories, Burlingame, CA), as described previously (Lehrer *et al.*, 1998). H^3TdR-positive cells were detected by autoradiography. Keratinocytes with intensive BrdU signal in the nuclei 8 weeks after injections were considered LRCs. Keratinocytes containing >5 silver grains over a nucleus were considered to be H^3TdR labeled. The number BrdU/H^3TdR double labeled keratinocytes was counted in skin sections, with bulge areas longitudinally sectioned through the center of the hair follicles (a total of 20–30 hair follicles from three individual animals/group).

DNA array and quantitative PCR analysis of GR expression in bulge keratinocytes

SC-enriched bulge keratinocytes were isolated by FACS analysis using CD34 and α6-integrin surface markers, as previously described (Trempus *et al.*, 2003; Morris *et al.*, 2004; Chebotaev *et al.*, 2007). Briefly, keratinocytes obtained from the dorsal skin of mice at the telogen stage of hair cycle were stained with FITC-conjugated rat anti-mouse α6-integrin Ab and biotin-conjugated rat anti-mouse CD34 Ab, followed by treatment with phycoerythrin-conjugated streptavidin (all reagents were from BD Pharmingen, San Jose, CA). α6-integrin$^+$/CD34$^+$ SC-enriched bulge keratinocytes and α6-integrin$^+$/CD34$^-$ basal keratinocytes were isolated using a MoFlo cell sorter (Dako, Fort Collins, CO), using Summit software (Dako), and purity of sorted cells was determined by post-sort FACS.

Total RNA was extracted from both keratinocyte populations, and purified by PicoPure™ RNA Isolation kit (Arcturus, Mountain View, CA). Quality of RNA was assessed by Bioanalyzer 2100 (Agilent Technologies Inc., Palo Alto, CA) and quantified spectrophotometrically (ND-1000, Nanodrop, Wilmington, DE). RNA samples from three individual sets of SCs and basal keratinocytes were used for two-round amplification and hybridization to Affymetrix Mouse 430 2.0 Arrays (Affymetrix, Santa Clara, CA). RNA amplification, labeling, hybridization, and data analysis were performed as described earlier (Chebotaev *et al.*, 2007). Robust probe-set summary of the log-transformed probe-level data was used to perform the *t*-test. Genes with a *P*-value$\leqslant$0.05 and a fold change $\geqslant$1.5 were considered statistically significant.

TaqMan real-time quantitative PCR was performed using ABI Prism 7900 (Applied Biosystems, Foster City, CA). Reverse transcription and amplification of total RNA (1–3 ng) from α6$^+$CD34$^+$ keratinocytes were accomplished in a one-step format with a TaqMan® One-Step real time reverse transcription PCR (RT–PCR) Master Mix Reagents kit (Applied Biosystems, Foster City, CA) and with predesigned/pre-optimized GR-specific TaqMan primers and probe from TaqMan® Gene Expression Assays kit with mouse GAPDH as endogenous control (Applied Biosystems, Foster City, CA).

Western blot analysis

To study the effect of FA on GR expression in epidermis, animals were treated with FA once, and killed 24 and 48 hours after the treatment. Epidermis was isolated from dermis by scraping on ice

with razor blade, and the whole cell protein extracts were prepared using radioimmunoprecipitation (RIPA) buffer, as described previously (Yemelyanov *et al.*, 2007). Proteins were resolved by SDS–PAGE on 10% gels, and transferred to nitrocellulose membranes (BioRad, Hercules, CA). Membranes were blocked with 5% Blotto in tris-buffered saline, and incubated with anti-GR polyclonal rabbit Ab (Santa-Cruz Biotechnology, Pasadena, CA) overnight at 4°C, followed by treatment with peroxidase-conjugated anti-rabbit IgG secondary Abs (Cell Signaling Technology, Beverly, MA). ECL reagent (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for the band visualization. To verify equal loading and adequate transfer, the membrane was probed with anti-α-actin Abs (Santa Cruz Biotechnology, Pasadena, CA).

Northern blotting

Animals were treated with FA using the intermittent regimen. Twenty-four hours after the first and fourth FA applications, epidermis was separated by scraping and total RNA was isolated from epidermis with TRI reagent (Molecular Research Center Inc., Cincinnati, OH). Northern blots (20 μg/lane) were probed for expression of *Mt1* as described previously (Budunova *et al.*, 1997). DNA probes were labeled by random primed reactions (Redipri-me™II, Amersham, Piscataway, NJ) using the complete coding sequence of murine *Mt1* (kind gift from Dr R. Lebovitz, Baylor College of Medicine, Houston, TX) as template.

Statistical analysis

There were three to four animals in each experimental group, and all experiments were repeated at least two times. Mean and standard error values for proliferation and morphometric analyses were calculated using Microsoft Excel software and compared using paired Student's *t*-test. Statistical analysis of GR expression in bulge and basal keratinocytes is described above.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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